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A sensitive surfactant-mediated spectrofluorimetric determination of chemotherapeutic agent topotecan in human serum and its investigation of DNA binding mechanism

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ABSTRACT: An overdose of an anticancer agent in the human body not only leads the high cytotoxicity on the neoplastic cells but also causes serious side effects. The regular detection of an anticancer agent level in biological fluids using an alternative technique is crucial in terms of assessment of therapeutic efficiency in chemotherapy process. In this work, we developed a micelle-enhanced spectrofluorimetric approach for the determination of topotecan (TPC), which is an effective anticancer agent used in the treatment of certain types of cancer, in human serum and binding mechanism of TPC-DNA. The proposed method exhibited a strong pH-dependent emission signal at 535 nm after excitation of 380 nm towards the TPC in the presence of surfactants. The relative fluorescence signal for TPC was found to be linear in the wide concentration range of 0.01 – 1.8 µM (R^2 = 0.9981) with a challenging detection limit of 3.3 nM. The developed spectrofluorimetric method was successfully applied to the analysis of TPC in spiked human serum samples with the good recovery results. Moreover, for the first time, the interaction mechanism between TPC and double-stranded DNA (ds-DNA) was studied by developed spectrofluorimetric method. The binding constant value of 8.5 x 10^{-3} M^{-1} calculated by Stern-Volmer method indicated the strong intercalation-based binding of TPC into the base pair of ds-DNA. The developed spectrofluorimetric method can provide new insight for the design of DNA-targeted drugs, and lead an alternative approach for the detection of anticancer drugs such as TPC in biological samples.

KEYWORDS: Spectrofluorimetry; Drug–DNA interaction; Fluorescence; Intercalation; Determination.

1. INTRODUCTION

Topotecan (TPC) is a semisynthetic and water-soluble analog structure of the plant alkaloid comptothecin, which specifically inhibits the intranuclear enzyme topoisomerase I activity. This inhibition causes a death of cancer cells by damaging the DNA strands fracture. Therefore, TPC is commonly used for the treatment of various cancer types including ovarian, breast and lung[1,2]. Although TPC exhibits a strong efficiency on the cancerous cells during chemotherapy process, its side effects such as hair loss, asthenia, immunosuppression and gastrointestinal diseases are seen as major challenges[3]. The quantification of TPC level in biological fluids is a highly desirable for the evaluation of chemotherapy process as well as regulation of side effects caused by TPC.

In last decades, considerable effort has been performed on the investigation of interaction mechanism of anticancer drugs with DNA. DNA is an important biological macromolecule that includes the genetic information for transcription. The interaction of anticancer agents with DNA may affect the cell growth and division or DNA replications by altering/inhibiting DNA functions[4,5]. Therefore, understanding the DNA-drug interaction mechanism is an exciting topic not only for the control of chemotherapeutic process, but also for the design of DNA-targeted drugs[6]. At this point, covalent (i.e. chemical modification) and non-covalent (i.e. electrostatic binding, groove binding or intercalation) binging modes have been commonly utilized in the characterization of small molecules binding into the DNA[5,7].

To date, various analytical techniques including spectrofluorimetry[8], high-performance liquid chromatography coupled with fluorescence[9] and tandem mass spectrometry detectors[10], and electrochemical sensors[11-13] have been proposed to detect the TPC in different media such as pharmaceutical drugs, blood or urine. Spectrofluorimetry has been extensively used for the determination of

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pharmaceutical compounds due to its sensitivity, selectivity, rapidity, easy-operation and low-cost[14,15]. T.G. Burke et al. developed a spectrofluorimetric method at two-different excitation photons for the detection of TPC analysis in blood samples[8]. However, the proposed spectrofluorimetric approach presents a low analytical performance towards the TPC owing to its lack of optimization parameters on the effect of fluorescence intensity of TPC.

The present work aimed to study the effect of fluorescence behavior of TPC in pH-dependent media including different type of surfactant, and its evaluation of analytical performance in human serum samples. Moreover, to the best of our knowledge, this work is the first spectrofluorimetric study to evaluate the binding mechanism between TPC and DNA, up to now. This developed spectrofluorimetric method could be a promising approach to determine the anticancer drugs such as TPC in biological samples, and evaluate its DNA interaction mechanism for further rational drug design applications.

2. RESULTS AND DİSCUSSİON

TPC has exhibited a well-defined native fluorescence at the emission band of 535 nm after excitation of 380 nm in aqueous solutions. This property could pave the way to develop a novel spectrofluorimetric approach for the determination of TPC without using any fluorescent derivative in pharmaceutical and biological samples. For this purpose, the effect of various experimental parameters including solvent type, pH of the media, surfactant and excitation wavelength on the fluorescence intensity of TPC was firstly studied, and the obtained results were presented below.

2.1.Optimization of experimental conditions

2.1.1. Effect of solvent type

Influence of the different solvents such as water, methanol, acetonitrile, acetone, DMSO and DMF on the fluorescence intensity of TPC was investigated, and the highest fluorescence intensity of TPC was observed in water due to the possible physical interaction strength between the solvent and excited singlet state of TPC. [14]

2.1.2. Effect of surfactant type and concentration

The micellar medium remarkably enhances the fluorescence intensity of a substance by increasing the molar absorptivity and fluorescence quantum yield of the fluorophore group. [16] Different surfactants such as CTAB as a cationic, SDS as an anionic and Tween-80 as a nonionic have been studied on the effect of fluorescence intensity of TPC. While the SDS decreases the fluorescence intensity of TPC compared to that of the absence of surfactant, CTAB and Tween-20 increased its fluorescence intensity. It is obviously seen from Figure 1a that the highest intensity for TPC was observed in the presence of CTAB, which is 1.1 higher than that of no surfactant. On the other hand, the influence of CTAB concentration in the range from 0.05% to 1.0% (w/v) on the fluorescence intensity of TPC was evaluated, and it was observed that the corresponding fluorescence intensity has increased with the increase of CTAB concentration from 0.05% to 0.2% and reached its maximum intensity at 0.2% CTAB. Then, the fluorescence intensity of TPC has decreased at higher CTAB concentrations (from 0.2 to 1.0%). Therefore, 0.2% CTAB was chosen as the optimum surfactant concentration for TPC analyzing (Figure 1b).

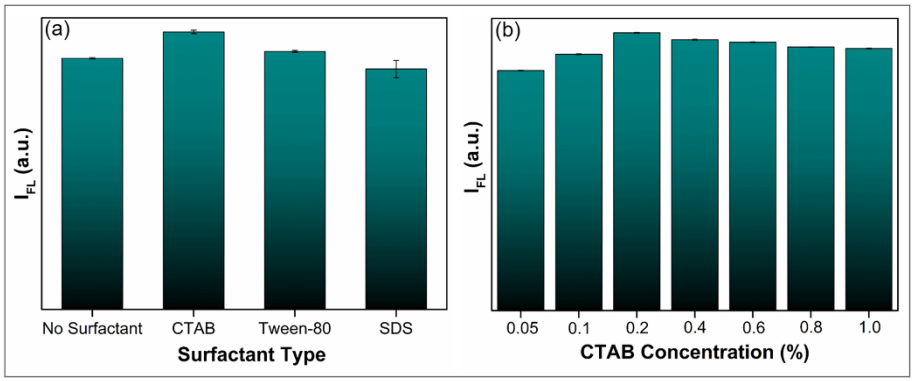

Figure 1. (a) Effect of various surfactants (0.5%) on the I_{FL} of TPC (1.0 µM) in aqueous media (n=3); (b) Effect of CTAB concentration (%w/v) on the I_{FL} of TPC (1.0 µM) (n=3).

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2.1.3. Effect of pH

Influence of pH on the native fluorescence intensity of TPC was investigated using different buffer media including phosphate and acetate buffer solutions in the pH range of 4.0-8.0 (Figure 2a). The fluorescence intensity of TPC has gradually increased with the increase of pH value from 4.0 to 7.0. Afterwards, the fluorescence intensity of TPC decreased when pH value shifts towards the more alkaline values. The maximum fluorescence intensity for TPC was achieved at pH 7.0, which is close to its pKa value.[17] Therefore, pH 7.0 phosphate buffer solution (PBS) is favored as an optimal pH value for the further fluorescence measurements.

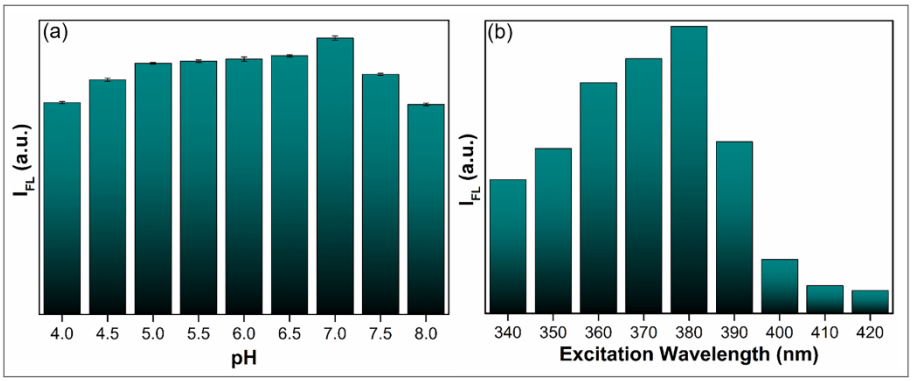

Figure 2. (a) Effect of pH on the I_{FL} of TPC (1.0 µM) in the presence of buffer solutions including 0.2% CTAB (n=3); (b) Effect of excitation of wavelength on the I_{FL} of TPC (1.0 µM) in pH 7.0 PBS including 0.2% CTAB.

2.1.4. Effect of excitation wavelength

The optimization of excitation wavelength is an essential parameter to increase the sensitivity of the spectrofluorimetric method. As seen in Figure 2b, the fluorescence intensity of TPC has gradually increased when the excitation wavelength increased up to 380 nm, and then remarkably decreased at higher excitation wavelengths. The maximum florescence intensity was observed at the emission band of 535 nm after excitation of 380 nm for 1.0 µM TPC in pH 7.0 PBS containing 0.2% CTAB.

2.2. Method Validation

The developed spectrofluorimetric method for TPC has been validated by performing linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision tests according to ICH guidelines.[18]

2.2.1. Analytical Performance

The analytical performance of the spectrofluorimetric method was firstly evaluated by determining its linear working range and detection limit under optimized experimental conditions (Media: pH 7.0 PBS including 0.2% CTAB, λExc: 380 nm). The developed spectrofluorimetric method exhibited a large linear working region in the range of 0.01 – 1.8 µM for TPC (Figure 3). The corresponding linear regression equation was expressed as I_{FL} = 482.83 C_{TPC} + 15.838, (R^2=0.9981). The LOD and LOQ values were calculated according to the following equations, respectively:

LOD: 3.3 SD/m; LOQ: 10 SD/m

Where SD is the standart deviation of the intercept of the linear calibration plot, and b is the slope of the calibration curve. The LOD and LOQ were estimated as 3.3 and 10.0 nM, respectively, confirming the sensitivity of the developed fluorimetric method for TPC.

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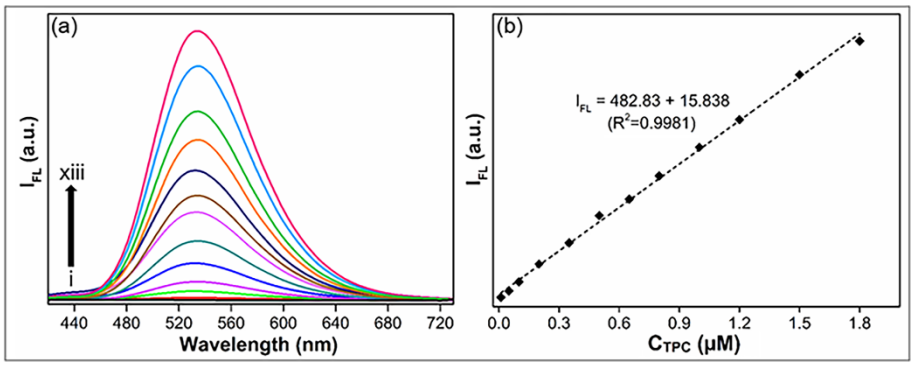

Figure 3. (a) Fluorescence spectra of TPC at various concentration in the range of 0.01 – 1.8 μM in pH 7.0 PBS containing 0.2% CTAB; (b) Calibration plot of I_{FL} *vs.* C_{TPC}.

Table 1. Analytical performance results obtained from spectrofluorimetric detection of TPC.

Parameters	TPC
λ_{ex} (nm)	380
λ_{em} (nm)	535
Concentration range (μM)	0.01 – 1.8
LOD (nM)	3.3
LOQ (nM)	10.0
Slope ± SD	482.83 ± 1.46
Intercept ± SD	15.838 ± 0.49
Correlation coefficient (R^2)	0.9981

* λ_{ex}: Excitation Wavelength, λ_{em}: Emission Wavelength, SD: Standard Deviation

2.2.2. Precision

The precision of the developed spectrofluorimetric method was evaluated by performing intraday and interday measurements of TPC at two different concentrations under optimized conditions. The intraday and interday results performed through five successive times and days, respectively, are summarized in Table 2. The low RSD values obtained indicate the acceptable precision of the developed spectrofluorimetric method for TPC.

Table 2. Precision results for TPC assay in a pure form obtained by spectrofluorimetric method.

Precision	Concentration (μM)	Found (μM)	Recovery (%)	RSD (%)
Intraday	0.5	0.514	102.87	0.22
	1.0	0.981	98.10	0.46
Interday	0.5	0.484	96.85	1.04
	1.0	0.991	99.14	1.41

2.2.3. Real Sample Applications

The validity of the developed spectrofluorimetric method for TPC has been performed using standard addition method in human blood serum samples. The serum samples were firstly spiked by known concentrations of TPC at three different levels, and then the plasma proteins in serum samples were precipitated by adding acetonitrile (1:1, v/v). Each amounts of the spiked TPC were successfully determined by developed spectrofluorimetric method under optimal experimental conditions. The average recoveries obtained were found to be in the range of 99.2 – 104.5% with the low relative standard deviation (RSD) values, which reveal the accuracy of developed spectrofluorimetric method towards the TPC assay (Table 3).

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Table 3. Application of the proposed spectrofluorimetric method for TPC assay in human blood serum.

Sample	Concentration (μM)	Found (μM)	Recovery (%)	RSD (%)
Blood Serum	0.1	0.099	99.2	0.61
	0.5	0.514	102.8	0.55
	1.0	1.045	104.5	0.38

2.3. DNA Interaction Mechanism

Fluorescence quenching is a useful strategy for assessing the interaction mechanism between drug molecules and DNA.[19,20] As seen in Figure 4, the fluorescence emission of TPC at 533 nm was linearly decreased by the increasing of DNA concentration up to 60 μM, indicating the binding of TPC into the double-stranded DNA via possible π-π stacking interaction.[20] On the other hand, a small red shift observed at the wavelength from 533 to 535 nm for TPC after the successive addition of DNA exhibits the intercalation-based binding of TPC to DNA helix structure. The intercalation-based binding mechanism of TPC with ds-DNA was further confirmed by determining the Stern-Volmer quenching constant (K_{sv}) value with the following equation [19,21]:

$$I_o/I = 1 + K_{SV}[DNA] \quad (1)$$

Where I_o and I are the fluorescence intensities of TPC in the absence and presence of DNA, respectively, and K_{SV} shows the quenching efficiency by DNA. K_{SV} value for TPC was calculated to be 8.5 x 10^{-3} M^{-1} from the slope of I_o/I *vs.* DNA concentration, suggesting the strong intercalation of TPC into the base pair of ds-DNA.

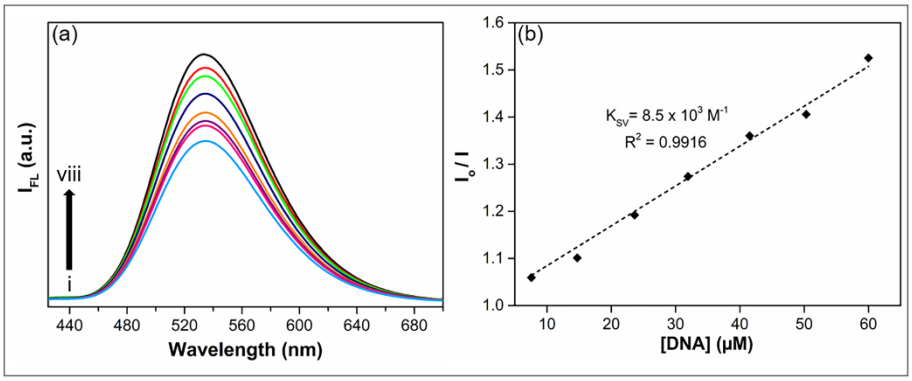

Figure 4. Fluorescence spectra of (i) TPC in the presence of ds-DNA at different concentrations (C_{TPC}: 1.0 μM, $C_{ds\text{-}DNA}$: (ii) 7.6, (iii) 14.7, (iv) 23.6, (v) 31.9, (vi) 41.5, (vii) 50.3 and (viii) 60.0 μM).

3. CONCLUSION

In conclusion, for the first time, a sensitive and effective surfactant-assisted spectrofluorimetric method has been developed for the determination of TPC in human serum samples. The developed spectrofluorimetric method exhibited a wide linear working range of 0.01 – 1.8 μM with a low detection limit of 3.3 nM. This method offers significant advantages including simple operation, fast response, cost-effective, high sensitivity and repeatability as well as no needing of pretreatment and derivatization process. The developed method has been successfully applied for the determination of TPC in human blood serum samples. The binding interaction mechanism of TPC with ds-DNA was also discussed by developed spectrofluorimetric method. Based on obtained results, this approach not only will benefit on the novel design of DNA-targeted drugs and but also enables to be used as an alternative tool for clinical applications.

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4. EXPERIMENTAL

4.1. Chemical and reagents

All reagents and solvents were of analytical grade, and used without further purification. Topotecan hydrochloride hydrate (98%), solvents including acetone, acetonitrile, dimethylsulfoxide, dimethylformamide, methanol and surfactants such as cetyltrimethylammonium bromide (98%), sodium dodecyl sulfate (99%) and liquid Tween-80® used in this study were purchased from either Sigma Aldrich or Alfa-Aesar Incs. Human blood serum samples were purchased from Sigma Aldrich Inc. and kept frozen at −20 °C until assay.

4.2. Apparatus

Fluorescence spectra were recorded using an Agilent Cary Eclipse spectrofluorometer (CA, USA) equipped with a Xenon flash lamp. The slit widths for both excitation and emission monochromators were set at 10 nm. All spectra ranging from 340 to 750 nm were measured in a 1.0 cm quartz cell at room temperature (25 °C). The pH measurements of all prepared solutions were carried out by a calibrated Mettler Toledo S220-K SevenCompact pH meter.

4.3. Preparation of standard TPC solutions

An accurately weighed TPC powder was carefully transferred into a 100 ml calibrated flask, and dissolved in methanol with the aid of ultrasonic bath to prepare a stock solution of 100 µM TPC. The prepared stock solution was further diluted with pH 7.0 PBS containing 0.2% CTAB to achieve the working solutions in the calibration range. All standard solutions were stored at 4 °C until their fluorescence measurements.

4.4. Preparation of spiked human blood serum samples

1 mL of blood serum sample was spiked with a known concentration of standard TPC at three different level, and followed by precipitating its protein content with acetonitrile using ultracentrifugation at 5000 rpm for 5 min. The supernatant of blood serum sample spiked with TPC was filtered through 0.45 µm PTFE membrane filter. A suitable amount of spiked serum sample was further diluted with pH 7.0 PBS containing 0.2% CTAB to be in the calibration range of TPC for fluorescence assay.

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